Effect of *Helicobacter pylori* infection on the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastric carcinogenesis in Mongolian gerbils

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The effect of *Helicobacter pylori* infection on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer was studied using a Mongolian gerbil model. Five-week-old male Mongolian gerbils were divided into four groups of 25–30 animals each and challenged for 20 weeks with *H.pylori*, MNNG, a combination of *H.pylori* and MNNG, or neither of them. Four to 20 animals from each group were killed at 16, 24, and 52 weeks after *H.pylori* inoculation, and histopathological changes in their stomachs were examined. A well-differentiated adenocarcinoma was first observed 24 weeks after inoculation in the combination group. At 52 weeks, only six of 15 animals were colonized with *H.pylori* persistently, and four of them showed well-differentiated adenocarcinomas; on the other hand, neither of the animals with disappearance of *H.pylori* from the combination group showed adenocarcinoma. At the same observation time, three of 17 animals from MNNG group showed poorly differentiated adenocarcinomas. The incidence of gastric carcinoma in the combination group was significantly higher than that in the MNNG group (P < 0.05). However, no tumors were seen in the control and *H.pylori* groups. The present findings demonstrate that *H.pylori* infection enhances the carcinogenic action of MNNG.

Introduction

Since the identification of *Helicobacter pylori* as the cause of chronic gastritis by Warren and Marshall in 1982 (1), the association between this microorganism and gastric cancer has been frequently reported (2–5). In 1994, the International Agency for Research on Cancer (IARC) conference of World Health Organization (WHO) defined *H.pylori* as a definite carcinogen in gastric cancer (6). Investigating this relationship in an experimental study, Fox *et al.* (7) reported that *Helicobacter mustelae* infection promotes *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced gastric cancer in ferrets. However, uninfected ferrets were not investigated in that report. Furthermore, *H.mustelae* differs morphologically and biochemically from *H.pylori* (8). Tsujii *et al.* (9) reported that gastric carcinoma occurred in rats receiving the ammonia solution, and hypothesized that ammonia produced by *H.pylori* is an important factor in *H.pylori*-related gastric carcinogenesis by increasing cell proliferation. Although suggestive, the above study does not demonstrate directly the relationship between *H.pylori* infection and gastric carcinoma. Recently, it has been reported that Mongolian gerbils were easily colonized with *H.pylori* and developed atrophic gastritis, intestinal metaplasia, and gastric carcinoma (10–13). However, the mechanism of carcinogenesis has not been clarified. A hypothesis that nitroso-compounds produced by anaerobes in the *H.pylori*-infected stomach play an important role in the gastric carcinogenesis has been advocated (14). A previous study demonstrated that MNNG induced adenocarcinoma in the glandular stomach of Mongolian gerbils (15).

In the present study, we aimed to investigate the effect of *H.pylori* infection on MNNG-induced gastric carcinogenesis in a Mongolian gerbil model.

Materials and methods

**Animals and chemicals**

Five-week-old male Mongolian gerbils (MGS/Sea; Seac Yoshitomi, Fukuoka, Japan) weighing 30–40 g were used. Animals were housed in stainless-steel cages on hard wood chips bedding in an air-conditioned room at 24°C. They were fasted for 24 h prior to *H.pylori* inoculation, and then fed with chow (Oriental Yeast Co., Tokyo, Japan) and distilled water ad libitum following 12 h of inoculation. MNNG (Alrich, Milwaukee, WI) was dissolved in distilled water at a concentration of 50 µg/ml and freshly prepared three times per week. It was given as drinking water ad libitum by light-shield bottle. Experiments were performed according to the guidelines of Ethical Committee for Animal Experiments at Oita Medical University (Oita, Japan).

**Organism**

*Helicobacter pylori* ATCC 43504, possessing the cagA gene and expressing vacuolating cytotoxin, was cultured on blood agar plates at 37°C under microaerophilic conditions for 4 days. The resultant colonies were inoculated in brucella broth (DIFCO Laboratories, Detroit, MI) with 10% horse serum at 37°C under microaerophilic conditions for 24 h. Inoculum size was then adjusted with sterile saline to produce the optical density of McFarland 4 at 540nm.

**Experimental design**

A total of 107 Mongolian gerbils were randomly assigned to four experimental groups (Figure 1). In group 1, 30 animals were fasted for 24 h and then given 1 ml of brucella broth containing 10% horse serum via i.g. tube. Twelve hours later, they were fed the free diet and distilled water. In group 2, 27 animals were fasted for 24 h and then challenged with 10⁸ c.f.u. of *H.pylori* in 1 ml of brucella broth with 10% horse serum. Twelve h later, they were fed as for group 1. In group 3, 27 animals were given MNNG for 20 weeks, from 4 weeks after the same treatment as group 1. In group 4, 25 animals were first treated the same as group 2, and at 4 weeks after that, the MNNG treatment was started. Subgroups of 4–20 animals from each experimental group were weighed and killed under anesthesia with ether at 16, 24 and 52 weeks after *H.pylori* inoculation. Sixty minutes prior to killing, a solution of 5-bromo-2'-deoxyuridine (BrdU; Sigma, St Louis, MO) was injected i.p. (100 µg/kg) into all animals. Immediately after killing, the blood was drawn from the heart, and the serum separated and stored at −20°C until use. The stomach, small intestine, colon, liver and lungs were quickly removed and used for histopathological examination.

**Histopathological examination**

Specimens of the excised organs were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E), Giemsa or immunohistochemical stain against *H.pylori*. Periodic-acid-Schiff or immunohistochemical stain against keratin was also applied when needed. For immunohistochemistry, the rabbit anti-*H.pylori* polyclonal antibody (DAKO, Kyoto, Japan) and the rabbit anti-human keratin antibody (DAKO) were used.
The animals successfully colonized with *H. pylori* showed the spiral bacteria in the mucous layer and gastric pits. In addition, they had also high IgG anti-*H. pylori* antibody titers (Table I). The infection rate of group 4 decreased from 100% at 16 weeks after inoculation to 40% at 24 and 52 weeks after inoculation, showing statistical significance (*P*, 0.01) (Table II). On the other hand, low titers of IgG anti-*H. pylori* antibody were observed in all animals from groups 1 and 3.

**Statistical analysis**

The results of the infection rate and the incidence of tumors were analyzed by the Fisher’s exact test or χ² test. The results of the serum antibody titer and the LI of anti-BrdU were expressed as means ± SD and analyzed for significance by the unpaired Student’s *t*-test or the Mann–Whitney *U* test. The *P* values <0.05 were considered significant.

**Results**

**Colonization with *H. pylori***

The animals successfully colonized with *H. pylori* showed the spiral bacteria in the mucous layer and gastric pits. In addition, they had also high IgG anti-*H. pylori* antibody titers (Table I). The infection rate of group 4 decreased from 100% at 16 weeks after inoculation to 40% at 24 and 52 weeks after inoculation, showing statistical significance (*P* < 0.01) (Table II). On the other hand, low titers of IgG anti-*H. pylori* antibody were observed in all animals from groups 1 and 3.

**Histopathological changes**

**Glandular stomach.** The infected animals from groups 2 and 4 revealed hyperplastic changes in the pyloric mucosa, cystic glandular dilation of the pyloric glands and erosions (Figure 2a). Some of the pyloric glands penetrated the muscularis mucosae multifocally and extended into the submucosa. Infiltration of lymphocytes, macrophages and neutrophils was seen in the lamina propria and submucosa with formation of lymphoid follicles. Atrophic gastritis and isolated goblet cell metaplasia were also observed in the pylorus, and they became severe gradually. In the early stage of infection, there was a slight inflammatory cell infiltration in the fundus, however, after 24 weeks, the ectopic pyloric glands with a severe inflammatory cell infiltration were observed in the fundic regions. In addition, the cell proliferation of the pyloric mucosa was markedly accelerated in the infected animals from groups 2 and 4 (Figure 2b; Table III). On the other hand, no abnormal findings were observed in animals from group 1 (Figure 2c and d), whereas a severe damage of the lamina propria mucosa
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**Fig. 2.** Photographs of the microscopic views of: (a) the pyloric mucosa from *H. pylori*-infected Mongolian gerbil showing hyperplastic changes with a marked inflammatory cell infiltration, the formation of lymphoid follicle, and extending pyloric glands into the submucosa (24 weeks, H&E, ×40); (b) the pyloric mucosa of the same *H. pylori*-infected animal showing an increased number of BrdU-labelled cells and a widespread of the proliferative zone (BrdU stain, ×200); (c) the normal mucosa of Mongolian gerbil from control group (52 weeks, H&E, ×100); (d) BrdU stain of the pyloric mucosa of the same Mongolian gerbil (BrdU stain, ×200); and (e) the pyloric mucosa from an animal treated with MNNG showing a severe mucosal damage with deformity (24 weeks, H&E, ×100).

with a defect of glands was found in animals from group 3 (Figure 2e).

*Forestomach.* Hyperkeratotic change and hypertrophy of the forestomach wall were observed in some MNNG-treated animals from groups 3 and 4. No abnormal findings were observed in animals from groups 1 and 2.
1264 cells were stained with keratin positively (Figure 3g), and regions was denuded. It was characterized by scant glandular atypia and infiltrated finely into the muscle layer. BrdU was stained positively not only in the proliferative neck region but of all animals. At 52 weeks (Figure 3e and f). The gastric wall in the region of group 4 was significantly higher than that in group 3 (P < 0.05) (Table IV).

(Continued)

**Discussion**

It has been reported that the Mongolian gerbil model is useful to study the relationship between *H. pylori* and gastric cancer, because *H. pylori*-infected animals showed atrophic gastritis and intestinal metaplasia (11). On the other hand, MNNG has been successfully used to produce gastric carcinoma in ferrets (7). It is becoming increasingly clear that multiple factors may be involved in the development of gastric carcinoma (7). Therefore, it seems important to study the effect of *H. pylori* infection on the carcinogenesis in MNNG-treated Mongolian gerbils.

In the present study, *H. pylori*-infection did not cause gastric cancer in Mongolian gerbils by 52 weeks after inoculation, whereas MNNG-treatment produced gastric cancer in three of 17 animals during the same observation period. When *H. pylori*-infected animals were challenged with MNNG, the incidence of adenocarcinoma significantly increased (four of six animals) versus the MNNG group. These findings suggested that *H. pylori* infection enhances the carcinogenetic action of MNNG.

In previous studies with *H. pylori*-infected Mongolian gerbils, no gastric cancer occurred by 52 weeks after inoculation (12,13). However, over a period of 1 year, observation of this model revealed the occurrence of an intestinal type of gastric cancer. Therefore, it can be said that while the development of intestinal type of gastric cancers seems to be a gradual process related to multiple carcinogenic factors such as dietary foods and/or intramucosal carcinogens, in addition to *H. pylori* infection (14), the chemical effect of MNNG advances the time of *H. pylori*-related gastric carcinogenesis.

Two main histological types of gastric carcinoma have been identified: intestinal and diffuse types (16). While the former has been associated with a sequential chain of events such as chronic gastritis, atrophy, intestinal metaplasia and dysplasia, this association has not been demonstrated for the latter (17). Watanabe et al. (12) reported that the well-differentiated adenocarcinoma in *H. pylori*-infected gerbils arises from intestinal metaplastic area. In the present study, there was a clear restriction of the histological types of adenocarcinoma to a particular group, with the well-differentiated adenocarcinoma restricted to group 4 and the poorly differentiated one restricted to group 3. Surprisingly, in contrast to the report of Watanabe et al. (12), well-differentiated adenocarcinomas in our study were not located in the intestinal metaplastic areas. These

**Table IV.** Occurrence of adenocarcinoma in Mongolian gerbils challenged with *H. pylori*, MNNG or *H. pylori* + MNNG

<table>
<thead>
<tr>
<th>Group</th>
<th><em>H. pylori</em> status</th>
<th>Incidence of adenocarcinoma, % (positive/total/time (weeks))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>–</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td>MNNG</td>
<td></td>
<td>0 (0/5)</td>
</tr>
<tr>
<td><em>H. pylori</em> + MNNG</td>
<td>+</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0 (0/3)</td>
</tr>
</tbody>
</table>

*a* *H. pylori* status was determined by histological and serological examinations. +, persistent positive; –, disappeared during experiment.

*b* *P* < 0.05 versus MNNG group.

**Table III.** Cell kinetics in the pyloric mucosa of Mongolian gerbils’ stomach

<table>
<thead>
<tr>
<th>Period (weeks)</th>
<th>Group (no.)</th>
<th>Labelling index of BrdU (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1 (5)</td>
<td>5.66 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>2 (5)</td>
<td>14.87 ± 7.51</td>
</tr>
<tr>
<td></td>
<td>3 (5)</td>
<td>7.90 ± 3.22</td>
</tr>
<tr>
<td></td>
<td>4 (5)</td>
<td>17.52 ± 8.16</td>
</tr>
<tr>
<td>24</td>
<td>1 (5)</td>
<td>6.20 ± 2.18</td>
</tr>
<tr>
<td></td>
<td>2 (4)</td>
<td>13.55 ± 6.03</td>
</tr>
<tr>
<td></td>
<td>3 (5)</td>
<td>6.97 ± 3.02</td>
</tr>
<tr>
<td></td>
<td>4 (2)</td>
<td>19.63 ± 9.05</td>
</tr>
<tr>
<td>52</td>
<td>1 (20)</td>
<td>6.45 ± 2.33</td>
</tr>
<tr>
<td></td>
<td>2 (14)</td>
<td>16.13 ± 7.88</td>
</tr>
<tr>
<td></td>
<td>3 (17)</td>
<td>8.87 ± 3.52</td>
</tr>
<tr>
<td></td>
<td>4 (6)</td>
<td>22.41 ± 8.04</td>
</tr>
</tbody>
</table>

*a* Group 1, uninfected control; group 2, infected with *H. pylori*; group 3, treated with MNNG; group 4, infected with *H. pylori* and treated with MNNG.

*b* Mean ± SD.

*c* *P* < 0.01 versus MNNG group.

Other organs. Formation of lymphoid follicles in the submucosa of duodenum and marked cystic changes of the duodenal glands were seen in some *H. pylori*-infected animals. No abnormal findings were observed in colon, liver and lungs from all animals.

Occurrence of carcinoma

Glandular stomach. At 24 weeks after inoculation, only two of five animals from group 4 were *H. pylori* positive, and one of them had a well-differentiated adenocarcinoma (Figure 3a and b). The tumor consisted of tubular structure with cellular atypia and infiltrated finely into the muscle layer. BrdU was stained positively not only in the proliferative neck region but also in the region of extending glands into the submucosa (Figure 3c and d). At 52 weeks, six of 15 animals of group 4 were *H. pylori* positive, and four of them had moderately or well-differentiated adenocarcinoma. In group 3, only three of 17 animals showed poorly differentiated adenocarcinomas at 52 weeks (Figure 3e and f). The gastric wall in the region of carcinoma became locally hypertrophic, though that in other regions was denuded. It was characterized by scant glandular structure with cellular atypia in the mucosa. Thus, carcinoma cells were stained with keratin positively (Figure 3g), and BrdU was accumulated in the proliferative zone of the mucosa only. On the other hand, no tumors were found in groups 1 and 2. All five tumors in group 4 invaded into the proper muscularis, whereas all three tumors in group 3 were localized to the lamina propria and submucosa. Neither metastasis nor invasion was observed in all animals with gastric carcinomas. At 52 weeks after inoculation, the incidence of gastric adenocarcinomas in group 4 was significantly higher than that in group 3 (P < 0.05) (Table IV).

Foregastro. Two animals from group 4 showed squamous cell carcinomas in the forestomach at 52 weeks after inoculation. One of them was persistently colonized with *H. pylori*, while the other one was *H. pylori* negative.

Other organs. No metastasis was observed in all animals with carcinomas.

Susceptibility of *H. pylori* to MNNG

The minimum inhibitory concentration of MNNG against *H. pylori* ATCC 43504 was 200 μg/ml, indicating that MNNG had a low anti-*H. pylori* activity.

Leukopenia

MNNG, *H. pylori* or *H. pylori* infected animals showed atrophic gastritis and intestinal metaplasia (11). On the other hand, MNNG has been successfully used to produce gastric carcinoma in ferrets (7). It is becoming increasingly clear that multiple factors may be involved in the development of gastric carcinoma (7). Therefore, it seems important to study the effect of *H. pylori* infection on the carcinogenesis in MNNG-treated Mongolian gerbils.

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Effect of *H. pylori* on MNNG-induced gastric cancer

Fig. 3. Photographs of the microscopic views of: (a) the well-differentiated adenocarcinoma in the pylorus of Mongolian gerbil infected with *H. pylori* and treated with MNNG at 24 weeks (H&E, ×40); (b) infiltration of tubular glands with cellular atypia into muscle layer, higher magnification of (a) (×400); (c) region of border among mucosa and submucosa in (a) (H&E, ×200); (d) positive staining with BrdU showing pyloric gland extending into the submucosa (BrdU stain, ×200); (e) the poorly differentiated adenocarcinoma in the pylorus of Mongolian gerbil treated with MNNG at 52 weeks (H&E, ×100); (f) structure with scant glandular formation showing in the mucosa, higher magnification of (e) (×400); (g) carcinoma cells in the mucosa showing positive staining with keratin (keratin immunohistochemical stain, ×400).
different findings in the two studies may be related to the different *H.pylori* strains used: a strain isolated from a patient with gastric ulcer in the report of Watanabe et al. (12) and a standard strain in the present study. In addition, we also used MNNG in our study.

Cellular susceptibility to topical MNNG is related to both gastric physiology and the cell cycle (7), and gastric epithelial cell proliferation is one of the important factors of gastric carcinogenesis (9,18,19). It has been reported that gastric mucosal cell proliferation is significantly higher in patients with *H.pylori*-related gastritis than in those with unrelated gastritis (20). In this study, the hyperplastic changes with cystic glandular dilation were seen in the pylorus of *H.pylori*-infected gerbils from groups 2 and 4. In addition, the proliferating cells were accumulated not only in the proliferative neck region but also in the glands extending into the submucosa. It might be thought that *H.pylori*-related hyperplastic changes participate in the genesis of well-differentiated carcinomas, and that such changes might be more strongly influenced by the carcinogenic action of MNNG compared with the mucosa without hyperplasia.

It has been reported that *H.pylori* persistently colonized the stomach of *N*-methyl-*N*-nitrosourea-treated Mongolian gerbils (21). In our study, the infection rate with *H.pylori* in group 4 decreased with time, and MNNG showed some anti-microbial activity against *H.pylori*. These findings suggested that the protracted MNNG-treatment may have contributed to the eradication of *H.pylori*. Interestingly, no carcinoma was found in the animals from group 4 with disappearance of *H.pylori*. Therefore, it is speculated that persistent *H.pylori*-infection has a positive effect on gastric carcinogenesis.

In conclusion, the present study suggests that *H.pylori* infection promotes the carcinogenic action of MNNG in Mongolian gerbils. In addition, this model may be useful in clarifying the mechanisms by which *H.pylori* infection results in the development of gastric carcinoma.

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